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Day 4 Biopsy Improves Pregnancy Outcome Comparing to Day 3 Biopsy in Preimplantation Genetic Screening

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Abstract

Aim: Preimplantation genetic screening (PGS) is a routine procedure performed in many *in vitro* fertilization (IVF) clinics. Embryo biopsy is an invasive procedure, and it has long been recognised that this procedure can affect the subsequent growth and development of the embryo.

Materials and methods: In total, 38 cycles from 31 couples were included in this study. Day 3 biopsy was performed on 126 embryos of 18 patients; 20 patients chose day 4 biopsy with 150 embryos tested. All specimens were screened on a 24-chromosome comparative genomic hybridization (CGH) array.

Results: Of the embryos subjected to day 3 and day 4 biopsies, 22.2% (28/126) and 28.7% (43/150) were normal, demonstrating that our biopsy system has no obvious detrimental effect on compaction. Embryos were transferred on the mornings of day 4 and day 5. Compared with day 3 biopsy (4/13; 30.8%), the day 4 biopsy (7/16; 43.8%) procedure provides an improved pregnancy rate with embryo transfer in current IVF cycle.

Conclusions: We suggest that biopsy performance on day 4, to obtain genetic materials without compromising embryo viability, shows promise for successful PGS in IVF.

Keywords: *In vitro* fertilization; Intra-cytoplasmic sperm injection; Preimplantation genetic screening; Preimplantation genetic diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) are routine procedures performed in many *in vitro* fertilization (IVF) clinics. In patients with genetic or chromosomal abnormalities, PGD is an integral part of the IVF program. In addition, PGS is used to replace the fluorescence *in situ* hybridization (FISH) to exclude single-gene disorders and to examine the structure of genetic and numerical abnormalities.

The first study describing successful biopsy of a human embryo for PGD was performed in 3-day-old embryos, which consisted of 6–8 cleavage-stage cells [1-3]. Presently, biopsies of 8-cell blastomeres or blastocyst trophectoderm obtained on day 3 or 5 are performed in IVF laboratories worldwide [3-6]. Harper et al. reported that biopsy of blastocyst trophectoderm on day 5 or 6 is more effective than biopsy of cleavage-stage blastomeres on day 3 [7,8]. In addition a larger amount of genetic material can be retrieved from biopsy of blastocysts than from biopsy of cleavage-stage embryos [3].

A greater number of cells facilitates genetic analysis, provides more results that are accurate and aid in detection of genetic and chromosomal abnormalities by using FISH, polymerase chain reaction (PCR), and comparative genomic hybridization (CGH) methods [9]. In addition, embryos biopsied on day 5 post fertilization have passed the compaction and cavitation passes of mammalian preimplantation development. Therefore, these embryos have the highest implantation potential [10].

Several recent studies have also shown that the rate of aneuploidy is significantly lower in blastocysts than in cleavage-stage embryos [11-14]. Additionally, Scott et al. reported that a biopsy performed on day 3 cleavage-stage embryos is more damaging than one performed on blastocysts [15]. The efficacy of PGS remains controversial. Currently there is a trend to move from day 3 to day 5/6 biopsy. However genetic screening of blastocysts can only be performed several hours to a day before embryo transfer, which can result in the cancellation of embryo transfer during the current IVF cycle, blastocyst cryopreservation, and embryo transfer in the next IVF cycle [11,16,17].

Recently, day 4 human embryos at the morula-stage were successfully biopsied. Elena et al. reported that day 4 biopsy procedure does not adversely affect embryo development [18]. However, few data have been published on day 4 biopsy of human morula-stage embryos. Here, we argue that biopsy on day 4 has the same benefits as biopsy on day 3, and can be more clinically useful. In this study, we present results from 38 IVF/ICSI (intra-cytoplasmic sperm injection) cycles in which PGS was performed on day 3 or day 4. In addition, we present data on clinical pregnancy outcomes after PGS. In 2010, Harper et al. estimated that approximately 90% of *in vitro* fertilization (IVF) clinics perform embryo biopsy and PGD on day 3 of embryos development, when the embryo is typically composed of 6–8 blastomeres [7].

Here, we argue that compact morula-stage biopsy on day 4 has the same benefits as biopsy on day 3 and can be more clinically useful. In this study, we present data from 38 IVF cycles with PGS for chromosome rearrangements, which were performed at our clinic and referring clinics from December 2013 to May 2015. To compare the effectiveness of day 3 and day 4 biopsies, we analysed embryo and chromosome data

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from a small cohort of PGS patients who elected either a day 3 biopsy with a fresh transfer or a day 4 biopsy.

Aim

The first aim of this study was to investigate whether the number of blastomeres biopsied from a day 3 embryo influenced the percentage of embryos with a conclusive PGS result. The second aim of this study was to determine the impact of day 3 and 4 biopsy on pregnancy outcome.

Materials and Methods

We retrospectively evaluated an analysis of 276 embryos from 38 ICSI/PGS cycles from 31 couples for chromosome rearrangement at our clinic from December 2013 to May 2015. We assigned the couples into two groups according to biopsy day: day 3 (18 cycles) and day 4 (20 cycles). All couples presenting for chromosome rearrangement testing had one or two blastomeres biopsied from their embryos, providing that the embryo had ≥ 8 blastomeres on day 3 and morulae blastomeres on day 4 post-oocyte collections.

The ICSI/PGS day 3 and day 4 groups did not differ significantly in age; patients were aged 37.2 ± 5.3 years and 37.1 ± 4.9 years, respectively. PGS was performed for couples with poor embryo implantation after conventional IVF, for couples experiencing infertility due to a chromosomal abnormality, and for couples with a history of recurrent miscarriages. All patients signed an informed consent form for ICSI/PGS that included counselling on the IVF program, risk of ovarian hyperstimulation syndrome, probability of pregnancy, risk of pregnancy complications, necessity of a prenatal diagnosis, and possible cryopreservation of supernumerary embryos obtained during the program.

Patients were subjected to ovarian stimulation with the use of gonadotropins (follicle-stimulating hormone or human menopausal gonadotropin), and gonadotropin-releasing hormone analogues or antagonists were used for controlled ovarian hyperstimulation. Patients received human chorionic gonadotropin when the diameter of the two or three leading follicles was >18 mm. Ultrasound-guided oocyte retrieval was performed 36 hours after the human chorionic gonadotropin injection, and luteal support consisting of vaginally administered progesterone was provided. Oocytes at MII were microinjected with ejaculated spermatozoa. Embryos were cultured before and after biopsy by using standard embryo culture conditions in our laboratory. Fertilized embryos were cultured at 37˚C, 6% CO_2, and 5% O_2 in culture medium (Quinn's Advantage Cleavage/Blastocyst medium, SAGE, Trumbull, CT, USA) supplemented with 10% serum protein substitute (SPS, SAGE) changed on days 3 and day 5. Approximately 16 - 20 hours after the procedure, fertilization was confirmed by the presence of two pronuclei and extrusion of the second polar body. In total 38 PGS cycles were included in the study. Day 3 biopsy was performed on 126 embryos from 18cycles, and day 4 biopsy was performed on 150 embryos from 20 cycles.

All specimens were screened on a 24-chromosome single nucleotide polymorphism (SNP) array. Biopsy and diagnosis was performed om 3 day cleavage-stage embryos and morula-stage embryos after fertilized embryos without signs of severe fragmentation and arrest were incubated in HEPES-buffered Ca^{2+}/Mg^{2+}-free biopsy medium (Ca^{2+}/Mg^{2+}-free biopsy medium, SAGE) for 30 min. In all cases, a Nikon TE 2000 inverted microscope (Tokyo, Japan), equipped with a Narishige NT-88 3D hydraulic micromanipulator (Tokyo, Japan), and different cutting instruments were used as follows (Figure 1).

Embryos were de-compacted in Ca^{2+}/Mg^{2+}-free biopsy media (SAGE). A hole was drilled in the zona pellucida using a ZILOS laser (Hamilton Thorne Research, Beverly, MA, USA) and one or two blastomeres gently aspirated for PGS analysis. The embryo was washed in blastocyst culture media (Quinn's advantage blastocyst culture medium, SAGE) and transferred into a fresh drop of media for ongoing culture.

The biopsied cells were washed and collected into a PCR tube with 2.5 µl of phosphate buffered saline (PBS, Irvine Scientific, Santa Ana, CA, USA). Extraction and amplification of DNA from the biopsied cells were performed according to the MG Flex single cell WGA (Whole genome amplification) kit system protocol (MG Flex; MG Med, Seoul, Korea). Approximately 3 µg of amplified DNA was used in the array CGH experiments (MG MED). Briefly, the amplified DNA was labelled with Cy-3 and Cy-5 dCTP for 3 hours by using a random priming method. The labelled DNA was purified, dissolved in hybridization buffer, and hybridized overnight. The slides were washed several times and dried. Images of the slides were acquired with a GenePix 4000B dual-laser scanner (Axon Instruments, Union City, CA, USA) and analysed with MGViewer analysis software (MG MED).

If embryos were considered normal, one or two embryos were transferred on day 4 or 5. Supernumerary unaffected embryos were cryopreserved. A pregnancy test was performed 13 days after embryo transfer. All women with a positive test underwent a transvaginal ultrasound scan two weeks after the positive test.

Statistical Analysis

Analyses were conducted using SPSS version 22. The mean, standard deviation, median, and quartiles of distribution were determined for each continuous variable. Pearson's chi-squared test (<0.05) was used to determine the distribution of different parameters. The Fisher's exact test was used to analyze the 2×2 contingency table.

Results

Between December 2013 to May 2015, we performed 38 PGS cycles for 31 couples (18 day 3 and 20 day 4 cycles) undergoing IVF. Patient characteristics such as mean maternal age, infertility duration, number of previous IVF cycles, number of oocytes retrieved, number of 2PN

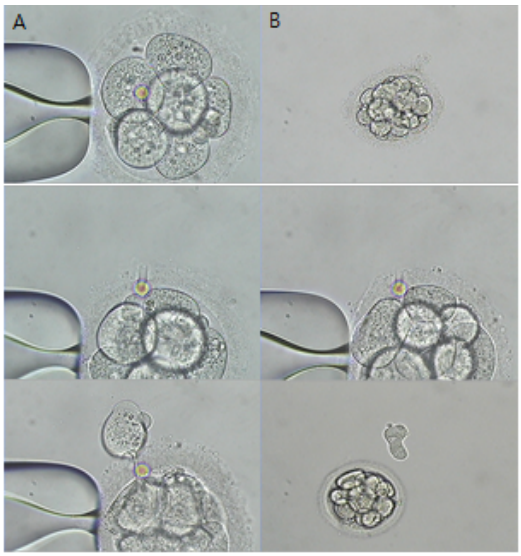

Figure 1: Cells obtained by biopsy for PGS. (A) Day 3 biopsy embryo. (B) Day 4 biopsy embryo.

(two pronucleus), number of PGS cycles, total number of embryo biopsied, and pregnancy rates are summarized in Tables 1 and 2. There were no differences between the two groups were observed regarding the age of the female partner, the duration of infertility, and the number of previous IVF cycles (Table 1).

Regarding the causes of infertility, 16 cycles (42.1%) had repetitive spontaneous abortion, 9 cycles (23.7%) had repetitive implantation failure, and 13 cycles (34.2%) had chromosomal abnormalities (Table 1). Of the total embryos (276/276:100%) successfully biopsied, aneuploidy was found in 76.2% (96/126) of day 3 and 71.3% (107/150) of day 4 embryos. Khi^2=3.706; p=0.157. In nine cases (9/38), transfer was not possible because all analysed embryos showed chromosomal abnormalities. Only two (2/276:0.7%) embryos were not diagnosed (Table 2). Similar number and frequency of embryos transferred on days 4 and 5.

Of the 29 cycles in which embryos were transferred after PGS, 11 were positive for hCG (human chorionic gonadotrophin) (37.9%) (Table 2). The day 3 and day 4 groups did not differ significantly in pregnancy rates (30.8% vs. 43.8%) (Khi^2=0.513; Φ=0.133; p=0.474), likely because the sample size was small. However, the pregnancy rate for day 4 biopsy embryos was higher than that of day 3 embryos. In the day 3 group, 4 patients tested positive for hCG (30.8%). The delivery rate per transfer was 15.4% (1 singleton and 1 twin). One biochemical pregnancy (0.8%) and 1 miscarriage (0.8%) were observed. In the day 4 group, 7 patients tested positive for hCG (43.8%). The ongoing pregnancy rate per transfer was 31.3% (5 singletons). One biochemical pregnancy (6.3%) and 1 ectopic pregnancy (6.3%) were observed.

Discussion

Chromosomal breakage is a widespread phenomenon in preimplantation embryos, affecting at least 10% of day 3 cleavage-stage embryos [6]. Repeated spontaneous abortions (RSA) are the main reason for numerical chromosomal abnormalities. A controversial indication for PGS is improving pregnancy rates in women who require assisted reproductive technology (ART) for reasons other than genetic indications. In addition, PGS aneuploidy screening is offered to patients experiencing recurrent implantation failure (RIF), which is typically defined as three or more consecutive IVF cycles without a clinical pregnancy despite the good embryo quality. For the diagnosis of genetic defects in human preimplantation embryos, embryos could theoretically be biopsied at any stage between the 2-cell and blastocyst stages [1-6,19-22].

Although biopsies of 6–8 cell embryos or blastocysts are generally successful, Swanson et al. reported that compact morula-stage biopsy is considered impossible because at this stage, cells are closely compacted and adhesive to other cells, making viable biopsy extremely difficult

	Day 3	Day 4	P-value
No. of cycles	18	20	
Female age (yr)	37.2 ± 5.3	37.1 ± 4.9	0.068
Infertility duration (yr)	3.6 ± 2.4	3.8 ± 2.6	0.165
No. of previous IVF cycles	2.8 ± 2.3	3.3 ± 2.6	0.213
Infertility factor (cycles)			
RIF	3	6	
RSA	9	7	
Chromosome abnormal	6	7	
RIF, repeated implantation failureRSA, repeated spontaneous abortion Values are presented as mean±standard deviation or number (%) p-value: not statistically significant (>0.05)			

Table 1: Patient characteristics.

Methods	Day 3	Day 4	P-value
No. of cycles	18	20	
No. of transferred cycles	13	16	
No. of cancelled cycles after PGS(abnormal chromosome)	5	4	
No. of retrieved oocytes (M ± SD)	217 (12.1 ± 7.6)	253 (12.7 ± 6.6)	0.798
No. of ICSI oocytes	199 (11.1 ± 7.2)	234 (11.7 ± 6.2)	0.768
No. of 2PN after ICSI (%)	158/199 (79.4)	180/234 (76.9)	0.900
No. of biopsied embryos (M ± SD)	126 (7.0 ± 4.1)	150 (7.5 ± 4.0)	0.707
normal (%)	28 (22.2)	43 (28.7)	0.157
abnormal (%)	96 (76.2)	107 (71.3)	-
no signal (%)	2 (1.6)	-	-
No. of transferred embryos (M ± SD)	22 (1.6 ± 0.9)	34 (2.1 ± 1.0)	0.208
Pregnancies (%)	4/13 (30.8)	7/16 (43.8)	0.474
Chemical pregnancies (%)	1 (25)	1 (14.3)	-
Abortions (%)	1 (25)	-	-
Ectopic pregnancies (%)	-	1 (14.3)	-
Ongoing pregnancies (%)	-	5 (71.4)	-
Delivery (%)	2 (50)		
Values are presented as mean ± standard deviation or number (%) p-value: not statistically significant (P>0.05)			

Table 2: Comparison of clinical outcomes in the two groups.

[23]. The current study has shown that embryos biopsied on days 3 and 4 showed no significant differences in development, these findings are supported by the reports published by Van de Velde and Michiels et al. [8,10].

Goossens and De Vos et al. reported that the removal of more than one blastomere from a day 3 embryo has a detrimental effect on developmental potential and day 5 embryo quality, and subsequently on clinical outcomes [4,22,23]. In 1998, Pey demonstrated the artificial decompaction of murine morula by using calmodulin, which is known to be one of the primary downstream effectors for Ca^{2+} [24]. Alikani and Kobielak suggested that cell-cell contacts during morula compaction are mediated by uvomorulin (epithelial-cadherin), a transmembrane calcium-dependent cell adhesion glycoprotein that is anchored to the cytoskeleton through catenins [25,26]. Although Ca^{2+}-free culture medium is can be used for artificial decompaction of morulae, it might result in conformational changes only within the extracellular domain of E-cadherin. We also employed that Ca^{2+}/Mg^{2+} free culture medium induced decompaction of human cleavage-stage and morula-stage embryos, which facilitated successful biopsy for PGS.

According to the 12th annual data collection (Data XII) of the European Society of Human Reproduction and Embryology Preimplantation Genetic Diagnosis (ESHRE PGD) Consortium in 2009, of biopsies performed during PGD/PGS cycles in Europe, 83.3% were performed on cleavage-stage embryos, whereas only 0.1% were performed on blastocysts (6/6102) [5].

Elena et al. reported that the day 4 biopsy procedure does not adversely affect embryo development [19]. Based on the above results, morula-stage biopsy is technically equivalent to cleavage-stage biopsy, and it provides more cells than early cleavage blastomere biopsy.

The percentage of embryos that reach the blastocyst stage and the pregnancy rate are important for human embryo development in IVF. De Vos and Goossens et al. reported that the blastocyst rate was 47–60% after biopsy of 3-day-old human embryos [4,23], and Elena

et al. demonstrated that 91% of embryos reached the blastocyst stage by day 5 after biopsy of morula-stage embryos. In bovine embryos, Abolfazl reported that biopsy of bovine embryos at the pre-compacted morula stage did not adversely affect *in vitro* developmental potential, and that the morula was a stable stage for blastomere removal [27]. The pregnancy rate after biopsy of morulae-stage embryos was not significantly different from that of the without PGS group [19]. Thus, the day 4 biopsy procedure is efficient and safe for use on morula-stage embryos. The high rate of blastocyst formation observed following decompacted human morula biopsy in this study is in concordant with data obtained from precompacted bovine morula biopsy [28]. Although embryo biopsy earlier than day 3 (<8 blastomere) in human [15] and bovine [28] embryos reduces viability, this might be a consequence of the small number of cells available at the cleavage stage.

In general, embryos without severe genetic abnormities can successfully complete the first crucial stage of early embryogenesis [28-30]. According to the ESHRE PGD Consortium data collection I–XI, aneuploidy of sex chromosomes is detected in 44% of embryos at the cleavage stage [5]. Furthermore, oligonucleotide DNA microarray analysis in blastocysts showed that 6.5% of embryos show aneuploidy of sex chromosomes (8.6% of embryos according to our study) and 5% of blastocysts show aneuploidy of chromosome 21 [31]. In this study, we also compared several parameters between the day 3 and day 4 groups, and observed no differences (Tables 1 and 2). This study shows that biopsy of morula-stage embryos provides sufficient cellular material suitable for genetic diagnosis regardless of which molecular genetics method is used. There is sufficient time after biopsy to obtain PGS results for embryo transfer on day 5 and 6 in the current IVF cycle. Furthermore, most embryos reach the morulae and blastocyst stages, and the pregnancy rate is comparable to the rate observed in un-biopsied embryos.

Recent years, the rate of embryo aneuploidy is increasing with the increase in female age at reproduction. Thus, genetic evaluation of human embryos during IVF provides patients with the opportunity to significantly reduce the risk of delivering a child with a genetic abnormality and to improve pregnancy and implantation rates. For these reasons, embryo biopsy and fresh embryo transfer are routinely performed in PGS cases. As previously mentioned, the available time before embryo transfer for transportation of genetic samples to the reference laboratory and for performing the procedures required for diagnosis is limited. In this study, PGS biopsy on day 4 shows promise as a tool for use in IVF.

Conclusions

We conclude that biopsy performance on day 4 to obtain genetic materials shows promise for successful PGS in IVF cycle.

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